



McLachlan, Jennifer, Beattie, Elisabeth, Murphy, Michael P., Koh-Tan, Caline H.H., Olson, Erin, Beattie, Wendy, Dominiczak, Anna F., Nicklin, Stuart A., and Graham, Delyth (2014) Combined therapeutic benefit of mitochondria-targeted antioxidant, MitoQ10, and angiotensin receptor blocker, losartan, on cardiovascular function. *Journal of Hypertension*, 32 (3). pp. 555-564. ISSN 0263-6352

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Deposited on: 14 February 2014

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# Combined therapeutic benefit of mitochondria-targeted antioxidant, MitoQ<sub>10</sub>, and angiotensin receptor blocker, losartan, on cardiovascular function

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**Objective:** Mitochondria-derived reactive oxygen species (ROS) play important roles in the development of cardiovascular disease highlighting the need for novel targeted therapies. This study assessed the potential therapeutic benefit of combining the mitochondria-specific antioxidant, MitoQ<sub>10</sub>, with the low-dose angiotensin receptor blocker (ARB), losartan, on attenuation of hypertension and left ventricular hypertrophy. In parallel, we investigated the impact of MitoQ<sub>10</sub> on cardiac hypertrophy in a neonatal cardiomyocyte cell line.

**Methods and results:** Eight-week-old male stroke-prone spontaneously hypertensive rats (SHRSPs,  $n=8-11$ ) were treated with low-dose losartan (2.5 mg/kg per day); MitoQ<sub>10</sub> (500  $\mu$ mol/l); a combination of MitoQ<sub>10</sub> and losartan (M+L); or vehicle for 8 weeks. Systolic pressure and pulse pressure were significantly lower in M+L rats ( $167.1 \pm 2.9$  mmHg;  $50.2 \pm 2.05$  mmHg) than in untreated SHRSP ( $206.6 \pm 9$  mmHg,  $P<0.001$ ;  $63.7 \pm 2.7$  mmHg,  $P=0.001$ ) and demonstrated greater improvement than MitoQ<sub>10</sub> or low-dose losartan alone, as measured by radiotelemetry. Left ventricular mass index was significantly reduced from  $22.8 \pm 0.74$  to  $20.1 \pm 0.61$  mg/mm in the combination group ( $P<0.05$ ). Picrosirius red staining showed significantly reduced cardiac fibrosis in M+L rats ( $0.82 \pm 0.22$  A.U.) compared with control ( $5.94 \pm 1.35$  A.U.,  $P<0.01$ ). In H9c2 neonatal rat cardiomyocytes, MitoQ<sub>10</sub> significantly inhibited angiotensin II mediated hypertrophy in a dose-dependent manner (500 nmol/l MitoQ<sub>10</sub>  $153.7 \pm 3.1$  microns vs. angiotensin II  $200.1 \pm 3.6$  microns,  $P<0.001$ ).

**Conclusion:** Combining MitoQ<sub>10</sub> and low-dose losartan provides additive therapeutic benefit, significantly attenuating development of hypertension and reducing left ventricular hypertrophy. In addition, MitoQ<sub>10</sub> mediates a direct antihypertrophic effect on rat cardiomyocytes *in vitro*. MitoQ<sub>10</sub> has potential as a novel therapeutic intervention in conjunction with current antihypertensive drugs.

**Keywords:** cardiac fibrosis, cardiac hypertrophy, hypertension, mitochondria, reactive oxygen species

**Abbreviations:** A.U., arbitrary units; ACE, angiotensin-converting enzyme; AngII, angiotensin II; ARB, angiotensin

receptor blocker; AUC, area under curve; CMI, cardiac mass index; De, external diameter; Di, internal diameter; dTPP, decyl triphenylphosphonium; eGFR, estimated glomerular filtration rate; KCl, potassium chloride; L-NAME, N<sup>G</sup>-nitro-L-arginine methyl ester; LVMI, left ventricular mass index; M+L, MitoQ<sub>10</sub> and losartan; MRA, mesenteric resistance artery; NOS, nitric oxide synthase; ROS, reactive oxygen species; SHRSP, stroke prone spontaneously hypertensive rat

## INTRODUCTION

Accumulating evidence demonstrates that mitochondria-derived reactive oxygen species (ROS) play an important role in the development of cardiovascular disease [1,2]. Conventional antioxidants cannot impact on mitochondria-derived ROS owing to their limited accumulation within mitochondria, highlighting the need for targeted therapies. Previously, we demonstrated that oral administration of the mitochondria-targeted antioxidant MitoQ<sub>10</sub> attenuates the development of hypertension, improves endothelial nitric oxide bioavailability and reduces cardiac hypertrophy in the stroke-prone spontaneously hypertensive rat (SHRSP) [3]. Although the MitoQ<sub>10</sub>-mediated reduction in blood pressure was modest, it may represent an important novel therapeutic agent for resistant hypertension and end-organ damage if combined with established antihypertensive drugs. Resistant hypertension remains a common problem with an estimated prevalence of 20–30%, despite a three-drug regimen [4].

Journal of Hypertension 2014, 32:555–564

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Received 21 December 2012 Accepted 15 October 2013

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DOI:10.1097/HJH.0000000000000054

This suggests existence of blood pressure elevating mechanisms, which are not fully addressed by current antihypertensive therapies. Combining a mitochondria-targeted antioxidant such as MitoQ<sub>10</sub> with commonly used antihypertensive agents may provide additive effects against the morbidity and mortality associated with resistant hypertension.

Studies by Dai *et al.* [5,6] indicate a critical role of mitochondrial ROS in cardiac hypertrophy, fibrosis and failure strengthening the rationale for mitochondria-targeted drugs. Our previous studies suggest a direct effect of MitoQ<sub>10</sub> on cardiac hypertrophy, demonstrated by a significant reduction in cardiac mass index (CMI) despite relatively small blood pressure attenuation [3]. Similarly, direct blood pressure independent effects on cardiac hypertrophy have been demonstrated with low-dose angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) [7–9]. Combining MitoQ<sub>10</sub> with a low-dose commonly used antihypertensive agent may achieve additional risk reduction beyond blood pressure lowering by addressing both cytosolic and mitochondrial mechanisms of ROS generation. The aims of this study were to investigate the potential complementary effects of a combined therapeutic strategy on the development of hypertension and cardiac hypertrophy using the mitochondria-targeted antioxidant MitoQ<sub>10</sub> and a low-dose ARB, losartan. In parallel, we investigated the contribution of mitochondrial oxidative damage to cardiomyocyte hypertrophy *in vitro* using H9c2 neonatal cardiomyocytes. These studies suggest new therapeutic interventions for resistant hypertension and related organ damage in humans.

## MATERIALS AND METHODS

An expanded Methods section is available in the Online Data Supplement, <http://links.lww.com/HJH/A299>.

### In-vivo experimental procedures

An inbred colony of SHRSP has been maintained at the University of Glasgow since 1991. Animals were housed under 12 h light/dark cycles at ambient temperature and were maintained on normal rat chow (Rat and Mouse No. 1 maintenance diet, Special Diet Services). All studies were conducted in accordance with the Animals Scientific Procedures Act 1986. Eight-week-old male SHRSP (8–11 rats per group) were treated with low-dose losartan (2.5 mg/kg per day); MitoQ<sub>10</sub> (500 µmol/l); a combination of MitoQ<sub>10</sub> and losartan (M + L); or vehicle for 8 weeks. MitoQ<sub>10</sub> was administered in drinking water, and losartan potassium (Sigma-Aldrich, Gillingham, Dorset, UK) administered daily and mixed with highly palatable baby food. SBP was measured by tail-cuff plethysmography [10,11] for the first 4 weeks of study. At 12 weeks of age, rats were implanted with radiotelemetry probes (Dataquest IV telemetry system; Data Sciences International, St Paul, Minnesota, USA) for haemodynamic measurement over the final 4 weeks of treatment [3,12]. Metabolic cages were used for the measurement of volume intake, urine output and collection of 24-h urine samples from control and treated rats at 16 weeks of age. At sacrifice, blood samples were taken by cardiac puncture, followed by measurement of heart

weight, left ventricle as well as septum weight and kidney weight for the calculation of CMI, left ventricular mass index and renal mass index, respectively (CMI, LVMI, RMI corrected for tibia length). Heart apices were fixed in 10% formalin for histological fibrosis assessment by picrosirius red staining. Aortae were taken for organ bath pharmacology and liver samples snap-frozen in liquid nitrogen.

## Ex-vivo experimental procedures

### Organ bath pharmacology

Organ bath pharmacology was used as described previously [3,11] to test contractile responses in aorta from control and drug-treated SHRSP. Vessels (approximately 4 mm in length) were pretreated with potassium chloride (KCl, 100 mmol/l), followed by construction of cumulative concentration–response curves to phenylephrine (10 nmol/l to 10 µmol/l in the absence and presence of N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 100 µmol/l) to inhibit nitric oxide species (NOS). Consecutive dose–response curves were carried out in the same vessel, measured first in the absence of L-NAME, followed by two washes with Krebs' buffer and then in the presence of L-NAME. The difference in tension in the absence and presence of L-NAME provides a measure of basal NO bioavailability and was calculated over the full dose–response curve and expressed as area under the curve (AUC). In addition, the rings were preconstricted to the EC<sub>50</sub> of phenylephrine and a concentration–response curve for relaxation to carbachol (10 nmol/l to 10 µmol/l) was obtained to measure stimulated NO release for which AUC was calculated. Responses to phenylephrine were standardized against the initial contractile response to KCl.

### Histology

Five-micrometre paraffin-embedded sections of heart apex were cut and deparaffinized with two washes in HistoClear (Fisher Scientific, Loughborough, UK) followed by rehydration through an ethanol concentration gradient. Fibrosis was assessed using picrosirius red staining (Sigma-Aldrich) specific for collagen type I and III. Sections were incubated under dark conditions in 0.1% picrosirius red solution, washed, rehydrated and cover-slip mounted. A colour threshold application was used to measure the average intensity of picrosirius red stain in five sections per heart apex from 3–8 rats per treatment group (ImageProPlus 4.1; Media Cybernetics, Marlow, UK). This involved transformation of pixel values to optical density units using the Area of Interest selection tool and Macro from the ImageProPlus software [13].

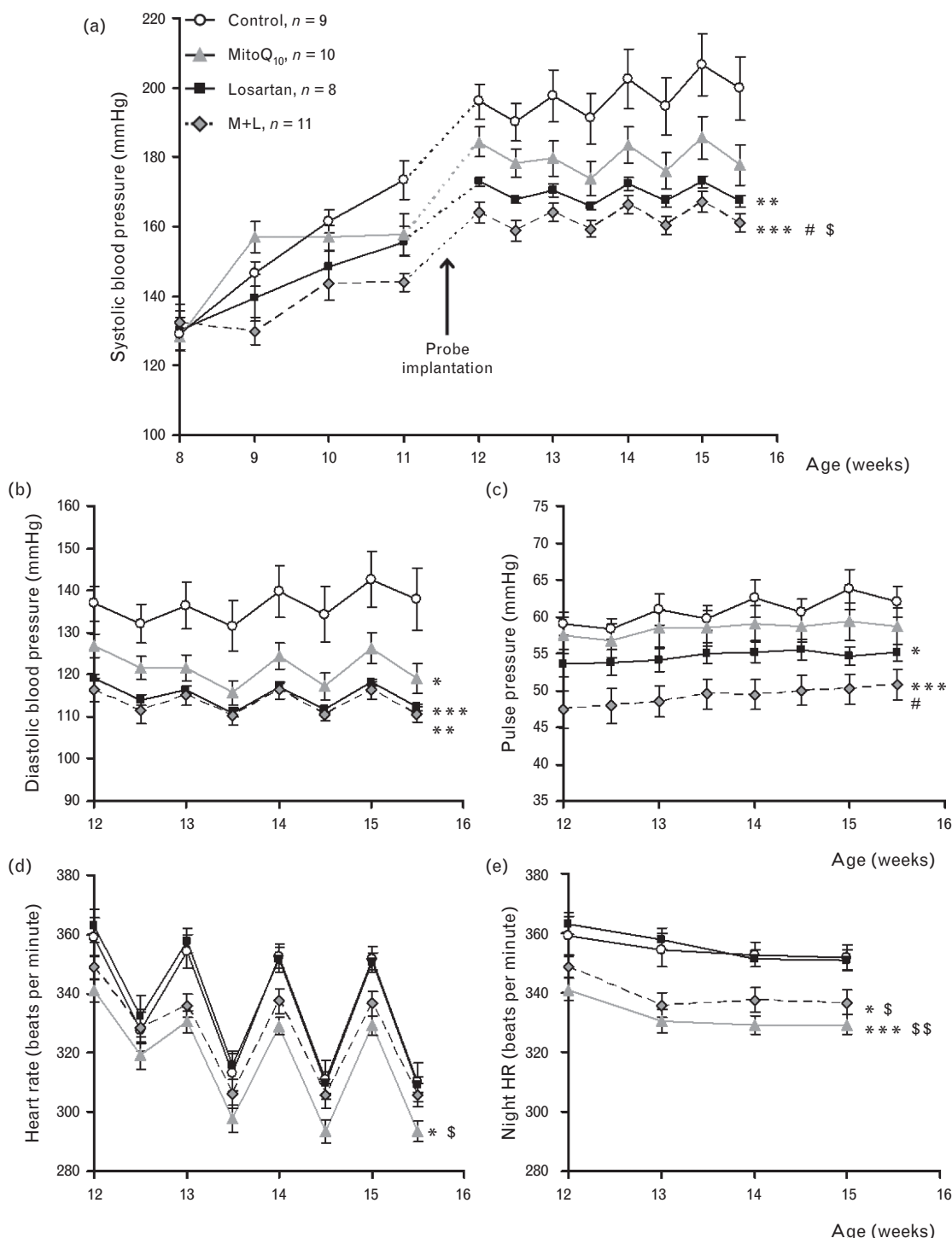
### Biochemical analysis

Urine sodium, potassium, urea and total protein levels were measured by routine biochemical analysis (Biochemistry Department, Gartnavel General Hospital, Glasgow, UK). Urinary and plasma creatinine were assessed for estimated glomerular filtration rate (eGFR) by the QuantiChrom Creatinine Assay Kit [Universal Biologicals (Cambridge) Ltd, Cambridge, UK] and urinary protein concentrations assessed by bicinchoninic acid (BCA) Protein Assay (Pierce,

Rockford, Illinois, USA). Lipid peroxidation in liver samples was determined by malondialdehyde (MDA) assay kit (Bioxytech LPO-586 Assay Kit; OxisResearch, Portland, Oregon, USA) according to manufacturer's instructions and normalized to protein concentration.

### In-vitro treatment of cardiomyocytes with MitoQ<sub>10</sub>

In-vitro assays were conducted in the H9c2 immortalized cardiomyocyte cell line derived from rat neonatal cardiomyocytes [14,15]. Cells were pretreated with MitoQ<sub>10</sub> or the



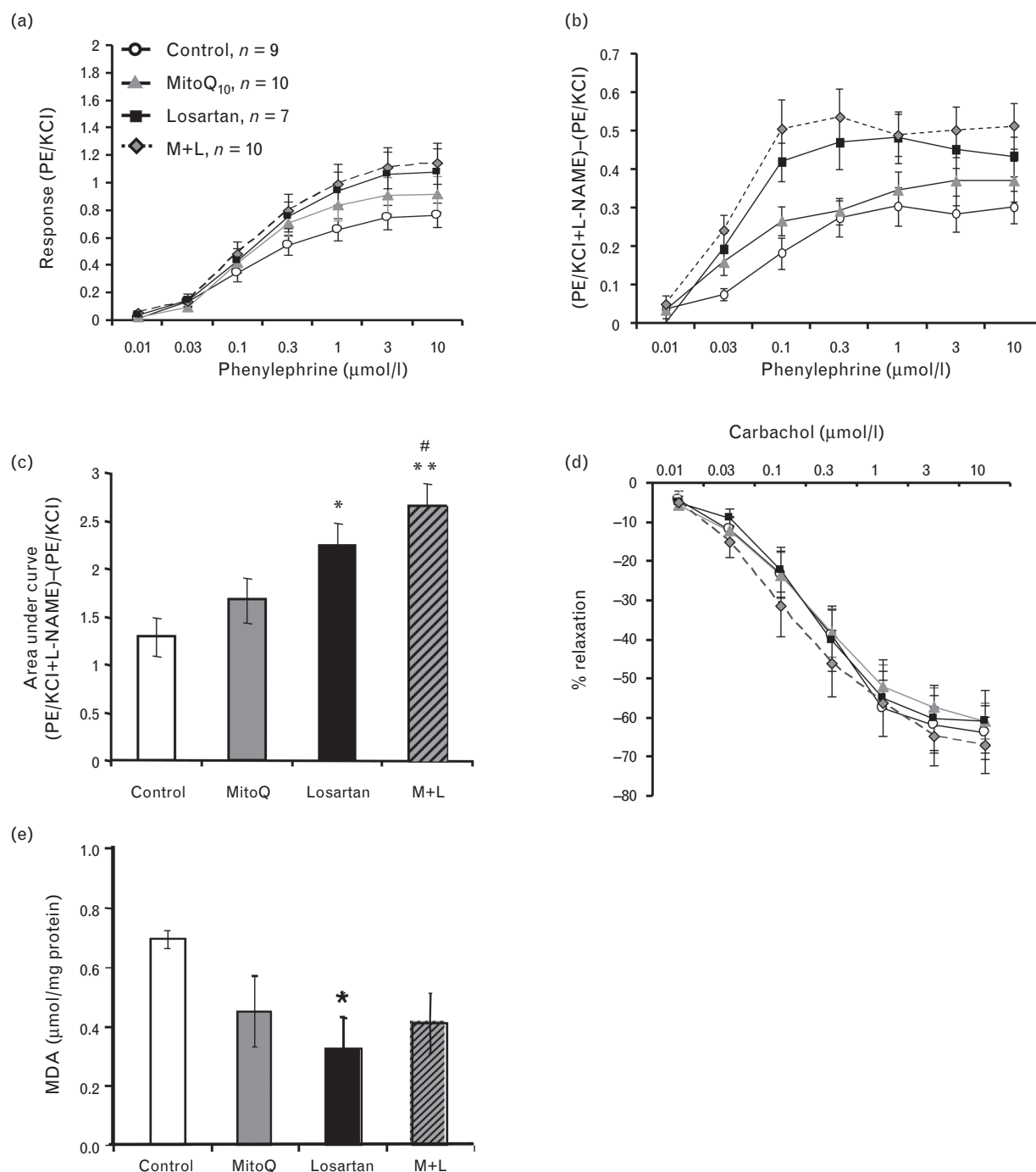
**FIGURE 1** Effect of MitoQ<sub>10</sub> treatment alone and in combination with low-dose losartan on the haemodynamic profile of SHRSP. (a) SBP measured by tail-cuff plethysmography and radiotelemetry was reduced by MitoQ<sub>10</sub> and losartan alone, and demonstrated greatest reduction with combined therapy (\*\* $P=0.004$ , \*\*\* $P<0.001$  vs. control; # $P=0.006$  vs. MitoQ<sub>10</sub> and  $^5P=0.05$  vs. losartan). (b) DBP was significantly reduced by individual and combination treatment (\* $P=0.032$ , \*\* $P=0.005$ , \*\*\* $P=0.003$  vs. control). (c) Pulse pressure shows small but significant reduction in losartan-only treated rats, and more pronounced reduction with combination therapy (\* $P=0.025$ , \*\*\* $P=0.001$  vs. control; # $P=0.01$  vs. MitoQ<sub>10</sub>,  $^5P=0.057$  vs. losartan only). (d) Heart rate measured by radiotelemetry was significantly reduced by MitoQ<sub>10</sub> (\* $P=0.012$  vs. control,  $^5P=0.001$  vs. losartan). (e) Night-time heart rate was significantly lowered by MitoQ<sub>10</sub> alone and by combination treatment compared with control and losartan only (\* $P=0.02$ , \*\*\* $P=0.001$  vs. control,  $^5P=0.006$ ,  $^5P<0.001$  vs. losartan only;  $n=8-11$ ).



control compound decyl triphenylphosphonium (dTPP) (10–500 nmol/l) for 18 h prior to angiotensin II (AngII) stimulation (100 nmol/l). After 96 h, cells were fixed with 2% paraformaldehyde, stained overnight with 2% crystal violet and cell size measured using ImageProPlus 4.1 software (Media Cybernetics). For each condition, 180 random cells were measured from 18 fields of view; experiments were repeated in triplicate on three independent occasions.

The MitoQ<sub>10</sub> analogue IBTP was colocalized to mitochondria using confocal microscopy as previously demonstrated (see supplementary methods, <http://links.lww.com/HJH/A299>) [16].

Cell Titre 96R Non-Radioactive Cell Proliferation Assay (Promega, Southampton, UK) was used according to manufacturer's instructions to test the cytotoxicity of MitoQ<sub>10</sub> and dTPP. A spectrometer (Wallac Victor2 plate-reader; Perkin



**FIGURE 2** Effect of MitoQ<sub>10</sub> alone and in combination with low-dose losartan on vascular function and oxidative stress. (a) Concentration response curves to PE stimulation. (b) Nitric oxide bioavailability measured as the difference in contraction to PE stimulation in the presence and absence of the NOS inhibitor L-NAME. (c) AUC calculated for basal NO bioavailability curves. Basal NO bioavailability was significantly increased in vessels from combination and losartan-treated rats (\**P* < 0.05 and \*\**P* < 0.01 vs. control. #*P* < 0.05 vs. MitoQ<sub>10</sub>; *n* = 7–10). (d) Relaxation to carbachol was not significantly different in vessels from control and treated rats. (e) Liver MDA was significantly reduced in losartan-treated rats (\**P* < 0.05, *n* = 5) with trends towards reduction in the MitoQ<sub>10</sub> and combination groups.

Elmer, Turku, Finland) measured absorbance of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 570 nm. Experiments were repeated in quadruplicate on three independent occasions.

## Statistical analysis

Results are expressed as mean  $\pm$  SEM. In-vivo experiments were performed with 8–11 rats per group. Repeated-measures analysis of variance (ANOVA) with Tukey pairwise comparison was used to compare radiotelemetry data. Ex-vivo comparisons were performed by one-way ANOVA with Tukey's multiple comparison test. In-vitro comparisons between groups were performed by one-way ANOVA with Bonferroni's multiple comparison test. Statistical significance was considered with *P* values less than 0.05.

## RESULTS

Average day-time SBP of control SHRSP increased from  $129.0 \pm 4.9$  mmHg to a maximum of  $206.6 \pm 9.0$  mmHg over the 8-week study (Fig. 1a). MitoQ<sub>10</sub> or low-dose losartan alone resulted in significant attenuation of SBP (MitoQ<sub>10</sub>;  $185.5 \pm 6.0$  mmHg, *P* = 0.032, Los;  $172.9 \pm 1.7$  mmHg, *P* = 0.004). When both drugs were used in combination, there was a highly significant reduction in SBP compared with control ( $167.1 \pm 2.9$  mmHg, *P* < 0.001), which was significantly lower than either treatment alone. DBP was significantly reduced by either drug alone (MitoQ<sub>10</sub>;  $126.3 \pm 3.7$  mmHg, *P* = 0.032, Los;  $118.2 \pm 0.77$  mmHg, *P* = 0.003) or in combination ( $116.3 \pm 2.1$  mmHg, *P* = 0.005) compared with control ( $142.6 \pm 6.5$  mmHg, Fig. 1b) but suggest no additive benefit of combined therapy. Pulse pressure was significantly reduced by combination treatment ( $50.2 \pm 2.1$  mmHg, *P* = 0.001) compared with control ( $63.7 \pm 2.7$  mmHg) and indicates additional benefit compared with either drug alone (Fig. 1c). Heart rate was significantly lower in MitoQ<sub>10</sub>-treated SHRSP ( $329.3 \pm 3.2$  bpm) than in control ( $351.6 \pm 4.3$  bpm, *P* = 0.012, Fig. 1d). Night-time heart rate, when the rats are most active, was significantly reduced in MitoQ<sub>10</sub> alone and combination treatment groups (Fig. 1e) compared with control or losartan only. Tibia length, fluid excretion/intake ratio and motor activity were not significantly different between groups (Supplementary figure i, <http://links.lww.com/HJH/A299>).

Basal nitric oxide bioavailability in aorta from control and drug-treated SHRSP is illustrated in Fig. 2a and

expressed as AUC in Fig. 2b. Basal nitric oxide bioavailability was significantly improved in losartan-treated SHRSP compared with control (*P* < 0.05) and in combination treatment compared with control (*P* < 0.05) and MitoQ<sub>10</sub> only (*P* < 0.05). Relaxation to carbachol was not significantly different in aortic rings from control and drug-treated SHRSP (Fig. 2c). Lipid peroxidation, measured by MDA assay, was used to assess oxidative stress in liver samples from control and drug-treated SHRSP (Fig. 2d). Treatment with losartan only resulted in a significant decrease in liver MDA levels (*P* < 0.05). The other treatment groups demonstrated similar trends but were not significantly different to untreated controls. No significant differences were observed for basal superoxide production in aortic rings or plasma lipid peroxidation (Supplementary Figure ii, <http://links.lww.com/HJH/A299>).

Kidney and urine characteristics for MitoQ<sub>10</sub>, losartan and combination-treated SHRSP were assessed (Table 1). There were no significant differences in kidney mass index, eGFR or urinary levels of Na<sup>+</sup>, K<sup>+</sup> or urea between control and treatment groups. However, total urinary protein excretion was significantly reduced in MitoQ<sub>10</sub>-treated rats (*P* < 0.05) with a similar trend in the combination therapy group.

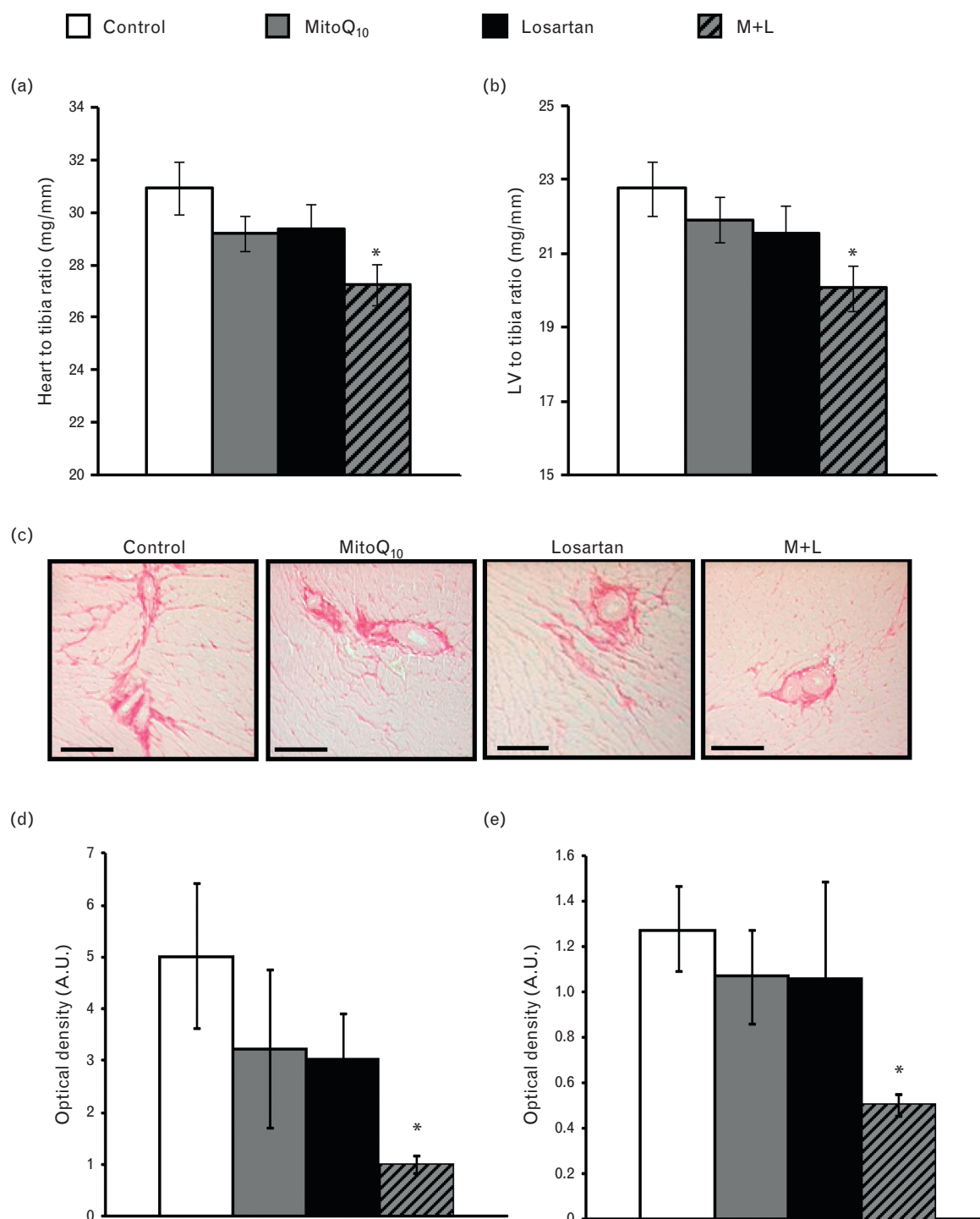
Combination treatment significantly reduced cardiac mass index (CMI) (M + L  $27.3 \pm 0.77$  mg/mm, control  $30.9 \pm 0.98$  mg/mm, *P* < 0.05) and LVMI (M + L  $20.1 \pm 0.61$  mg/mm, control  $22.8 \pm 0.74$  mg/mm; *P* < 0.05) compared with untreated controls (Fig. 3a, b). Cardiac fibrosis was assessed by picrosirius red staining (Fig. 3c) and collagen I and III immunohistochemistry (Supplementary Figure iii, <http://links.lww.com/HJH/A299>). Elevated levels of fibrillar collagen in perivascular and interstitial regions (Fig. 3c) in control hearts were significantly attenuated by combination treatment (*P* < 0.01) (Fig. 3d).

Exposure of H9c2 cardiomyocytes to AngII increased cell size from  $159.2 \pm 3.3$  to  $200.1 \pm 3.6$   $\mu$ m (*P* < 0.001) (Fig. 4a), and this was prevented by MitoQ<sub>10</sub> but not by the control compound dTPP (Fig. 4b). MitoQ<sub>10</sub> had no effect on cell size in unstimulated H9c2 cells and cell viability was not affected by MitoQ<sub>10</sub> or dTPP (Supplementary Figure iv, <http://links.lww.com/HJH/A299>). Immunofluorescence to detect the MitoQ<sub>10</sub> analogue IBTP in cardiomyocytes demonstrated uptake and colocalization to mitochondria during hypertrophy (Fig. 4c).

**TABLE 1. Effect of MitoQ<sub>10</sub> alone and in combination with low-dose losartan on renal parameters**

	Control	MitoQ <sub>10</sub>	Losartan	M + L
Kidney:tibia ratio (mg/mm)	$27.1 \pm 1.06$	$28.1 \pm 0.90$	$27.6 \pm 1.04$	$28.1 \pm 0.91$
eGFR (ml/min per g kidney weight)	$0.25 \pm 0.03$	$0.17 \pm 0.02$	$0.22 \pm 0.02$	$0.20 \pm 0.01$
Urinary protein (mg/ml per 24 h)	$120.7 \pm 15.4$	$75.8 \pm 10.5^*$	$110.5 \pm 8.0$	$83.6 \pm 7.7$
Urinary Na <sup>+</sup> (mmol/24 h)	$0.92 \pm 0.21$	$0.84 \pm 0.35$	$0.85 \pm 0.08$	$1.07 \pm 0.20$
Urinary K <sup>+</sup> (mmol/24 h)	$2.06 \pm 0.28$	$2.00 \pm 0.31$	$2.14 \pm 0.20$	$2.18 \pm 0.19$
Urea (mmol/24 h)	$6.99 \pm 0.54$	$4.92 \pm 1.36$	$5.74 \pm 1.26$	$6.02 \pm 0.41$

Values are means  $\pm$  SEM; *n* = 8–11 rats per group. eGFR, estimated glomerular filtration rate. \**P* < 0.05 compared with untreated control.



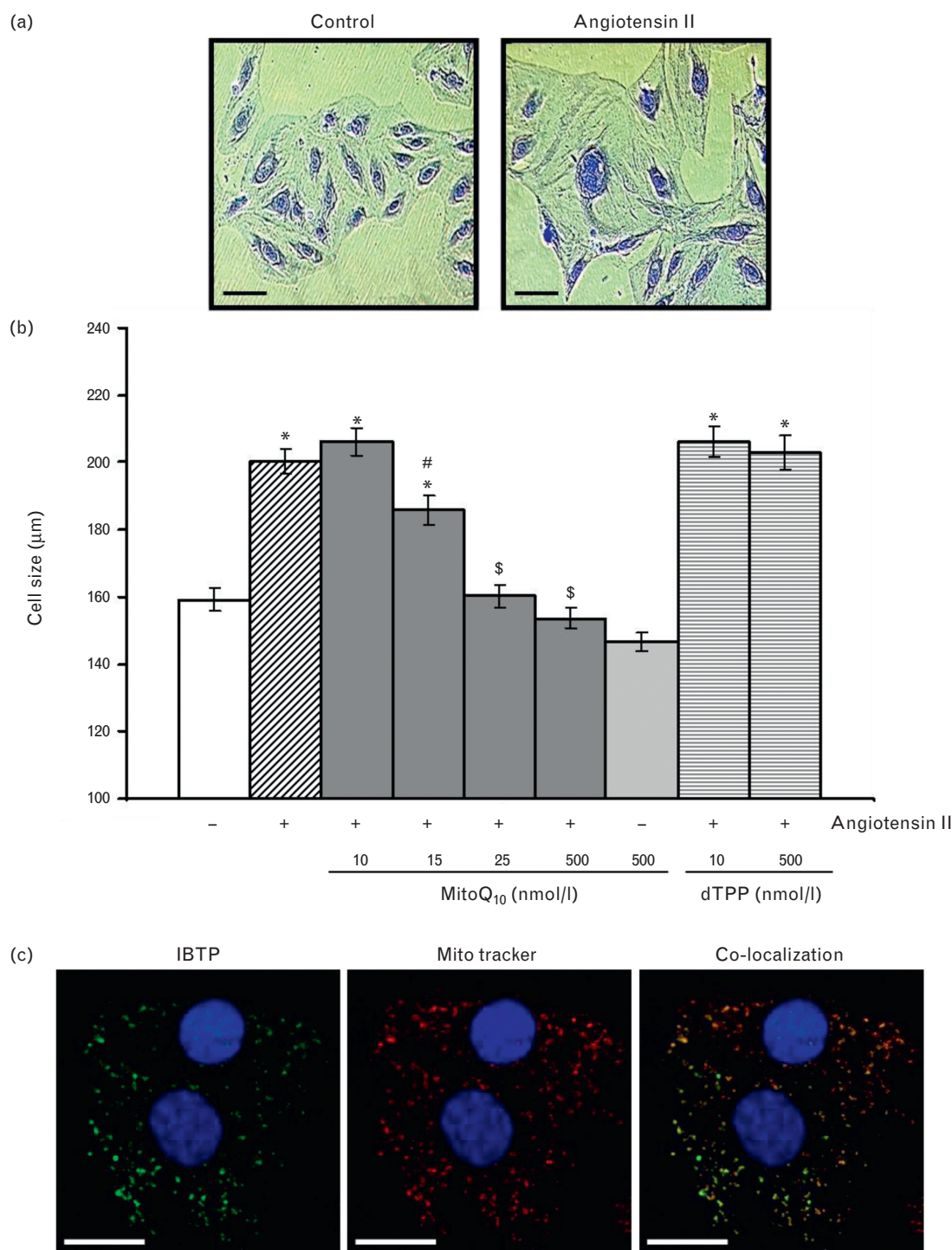
**FIGURE 3** Effect of MitoQ<sub>10</sub> alone and in combination with low-dose losartan on cardiac and left ventricular hypertrophy and fibrosis. (a) CMI and (b) LVMI were significantly reduced in combination-treated SHRSP compared with control ( $n=8-11$ ,  $*P<0.05$  vs. control). Single-drug therapy had no significant effect on CMI or LVMI. (c) Representative images of perivascular and interstitial cardiac fibrosis (magnification  $\times 10$ , scale bar = 100  $\mu\text{m}$ ) analysed with picrosirius red staining and quantified using ImageProPlus. (d) Perivascular and (e) interstitial fibrosis was significantly reduced in combination-treated SHRSP compared with controls ( $*P<0.05$  vs. control,  $n=3-8$ ).

## DISCUSSION

This study demonstrates that administration of MitoQ<sub>10</sub> provides complementary therapeutic benefit to an established antihypertensive agent. Our data suggest that MitoQ<sub>10</sub> targets elements of the hypertensive and hypertrophic processes involving mitochondrial oxidative

damage that are not fully addressed by current antihypertensive drugs. Furthermore, our in-vitro studies demonstrate that MitoQ<sub>10</sub> has a direct antihypertrophic action in cardiomyocytes indicating that its in-vivo action may be partly independent of blood pressure lowering.

We have previously demonstrated that the mitochondria-specific antioxidant MitoQ<sub>10</sub> has important



**FIGURE 4** Effect of MitoQ<sub>10</sub> on AngII-induced cardiomyocyte hypertrophy. (a) Representative images of control and AngII-stimulated H9c2 cardiomyocytes after 96 h incubation and crystal violet staining (magnification  $\times 10$ , scale bar = 100  $\mu\text{m}$ ). (b) MitoQ<sub>10</sub> prevented development of AngII-stimulated hypertrophy in a dose-dependent manner (\* $P < 0.001$  vs. control, # $P < 0.05$  and \$ $P < 0.001$  vs. 10 nmol/l MitoQ<sub>10</sub>), whereas dTPP had no effect. (c) The MitoQ<sub>10</sub> analogue, IBTP, was detected in cells after 96 h, co-localized with MitoTracker Red. Nuclei were counterstained with DAPI (magnification  $\times 63$ , scale bar = 50  $\mu\text{m}$ ).

antihypertensive action in young SHRSP [3] emphasizing a key role for mitochondrial-oxidative stress in the development of hypertension. Other in-vivo studies [17–21] have shown that MitoQ<sub>10</sub> is effective against mitochondrial oxidative damage in rodent models of sepsis, cardiac reperfusion, metabolic syndrome and diabetic nephropathy. Together, these findings highlight the importance of

targeted antioxidant therapy in a range of diseases affected by mitochondrial oxidative damage.

Several sources of ROS may contribute to the development of cardiovascular disease, including NADPH oxidase, xanthine oxidase, uncoupled nitric oxide synthase (NOS) and the mitochondrial electron transport chain [1,22–24]. There is a complex interplay between these different ROS

and the precise contributions to the underlying disease mechanisms remain obscure. There is evidence for ROS-induced ROS production, with mitochondria being both stimulated by NADPH oxidases and also acting as the initiating stimulus for further ROS generation [2,5,24–26], creating a potential feed-forward cycle of ROS production. In the present study, MitoQ<sub>10</sub> was tested in combination with a low-dose commonly prescribed ARB. This combined therapy demonstrated significant additive haemodynamic benefit, antihypertrophic and antifibrotic action when compared with control or single therapies. Only DBP failed to show additive reduction during combined versus single therapy, suggesting that MitoQ<sub>10</sub> and losartan share a common antihypertensive mechanism for diastolic pressure. The lack of an additive effect on DBP is potentially beneficial, as evidence suggests that therapeutically induced diastolic hypotension can be a risk factor for increased coronary events [27–29]. In line with the non-additive effects on DBP, we also observe similar significant equivalent improvements in aortic nitric oxide bioavailability in the combination and losartan-only group. The significant reduction in heart rate by MitoQ<sub>10</sub> treatment, either alone or in combination with losartan, during the active (night-time) period, was not observed with single losartan therapy. MitoQ<sub>10</sub> may alter heart rate by reducing oxidative stress within the rostral ventrolateral medulla (RVLM), as mitochondrial-derived ROS has been shown to mediate sympathoexcitation within this brain stem cardiovascular control centre [30]. We also demonstrate reduced urinary protein levels in MitoQ<sub>10</sub> and combination-treated rats indicating that mitochondria-specific antioxidants may also play a role in reducing end-organ damage in the kidney.

Increased ROS production stimulates myocardial fibrosis and is associated with reduced cardiac function [31]. This may be due to the accumulation of perivascular collagen compressing coronary arterioles and the accumulation of fibrillar collagen in the myocardial interstitium increasing wall stiffness and impairing cardiac systolic function [32]. Significant cardiac fibrosis has previously been reported in male SHRSP by 16 weeks of age [33,34], and in the current study, we demonstrate prevention of cardiac fibrosis by combination treatment. The design of the current study does not allow direct mechanistic assessment of the antifibrotic action of MitoQ<sub>10</sub>. This would require investigation in alternative models of cardiac fibrosis such as the transverse aortic constriction (TAC) model or the renal hypertensive rat (RHR), permitting pre and postsurgery assessment of MitoQ<sub>10</sub> action. However, we suggest that the significant reduction in collagen deposition by combination therapy is likely to be due to decreased oxidative damage. Losartan lowers blood pressure and decreases left ventricular hypertrophy by blocking AT<sub>1</sub> receptors, and reducing AngII-dependent activation of NADPH oxidases, [35,36]. This, in turn, can reduce mitochondrial-derived ROS production [24,26] in addition to the direct mitochondrial antioxidant potential of MitoQ<sub>10</sub>. Our data suggest that combining a mitochondria-targeted antioxidant with losartan may provide superior antihypertensive, antihypertrophic and antifibrotic action by interrupting the vicious cycle of ROS production and oxidative damage.

Although this study was unable to demonstrate direct MitoQ<sub>10</sub>-induced improvement in ROS, this was not entirely unexpected considering that O<sub>2</sub><sup>•−</sup>, the primary ROS produced by the mitochondria, is not readily diffusible across mitochondrial membranes and is largely dismutated to H<sub>2</sub>O<sub>2</sub> [37]. Moreover, it is well recognized that measurement of reactive molecules in biological environments is inherently challenging due to their short lifespan and the limited selectivity of detection systems [38–40].

Cardiac hypertrophy is an adaptive response to increased blood pressure, and as a result, practically all antihypertensive agents reduce LVMI, emphasizing the importance of haemodynamic load in the pathogenic process. However, convincing evidence from studies with low-dose ACE inhibitors and ARBs also demonstrate blood pressure independent attenuation of left ventricular hypertrophy [7–9]. The beneficial action of MitoQ<sub>10</sub> on cardiac hypertrophy despite modest blood pressure reduction suggests a direct role for mitochondrial oxidative stress in the hypertrophic process rather than simply a secondary effect of blood pressure reduction [3]. In support of this, H9c2 cardiomyocytes, a well established model of hypertrophy [14,15,41], showed dose-dependent inhibition of hypertrophy by MitoQ<sub>10</sub>. These data suggest a potentially important therapeutic role for MitoQ<sub>10</sub> in heart disease independent of its haemodynamic effects.

The current preclinical study has investigated the beneficial action of MitoQ<sub>10</sub> during the developmental of hypertension and left ventricular hypertrophy. The next stage will be to determine the effects of mitochondria-specific antioxidant efficacy in the more clinically relevant setting of established hypertension. These future studies will provide important information for potential clinical trials of human resistant hypertension, as MitoQ<sub>10</sub> was proven well tolerated in two phase II clinical trials [42,43].

## Clinical perspectives

Oxidative stress and mitochondrial dysfunction are increasingly implicated in cardiovascular disease and yet this is a neglected aspect in current treatment strategies. We demonstrate that MitoQ<sub>10</sub> is a potential complementary therapeutic intervention to current antihypertensive agents, particularly in the treatment of haemodynamic changes and end-organ damage associated with resistant hypertension.

## ACKNOWLEDGEMENTS

British Heart Foundation Chair (CH98001) and Programme grant funding (RG/07/005), EU Sixth Framework Programme Integrated Project (LSHG\_CT 2005-019015 EURA-Tools), EU Community's Seventh Framework Programme (FP7/2007–2013) under grant agreement (HEALTH-F4–2010–241504 EURATRANS) awarded to A.F.D. BHF PhD studentship (FS/07/029/23022) and project grant (PG/11/82/29136) awarded to D.G. and A.F.D.

## Conflicts of interest

M.P.M. is on the scientific advisory board of, and holds shares in, Antipodean Pharmaceuticals Inc, which is commercializing MitoQ<sub>10</sub>.

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## Reviewers' Summary Evaluations

### Referee 1

The manuscript explores the efficacy of MitoQ10 as a combination therapy with an angiotensin receptor blocker in reducing cardiac fibrosis with hypertension. Although, there are several antihypertensives that can be used clinically to prolong survival in hypertension induced heart failure, there is still a need to find better therapeutic molecules. One of the possible targets is reducing the reactive oxygen species generated by mitochondria thus limiting cardiac fibrosis and the consequent heart failure. Among several antioxidants tested so far, none has been shown to be effective. Something like MitoQ<sub>10</sub> may provide a benefit as combination therapy, provided the animal experiments are confirmed in humans.

### Referee 2

There is convincing evidence that oxidative stress is involved in the pathogenesis of hypertension but data from clinical trials with antioxidants are inconsistent. Targeting the enzymes responsible for reactive oxygen species (ROS) generation could be an effective strategy. Mitochondria is an important source of ROS at cardiovascular level. This study shows that the combination of the mitochondria-targeted antioxidant MitoQ<sub>10</sub> and losartan provides additive effects, attenuating hypertension and left ventricular hypertrophy of stroke-prone spontaneously hypertensive rats. Thus, mitochondria-targeted antioxidants would provide complementary therapeutic benefit to the inhibitors of renin-angiotensin-system in the treatment of hypertension. However, the clinically relevance of the observed effects in this work are unknown.